ADVANCED CATALYTIC ENZYME SYSTEM (ACES) – DUAL USE CAPABILITIES

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ABSTRACT

An enzyme-based decontaminant (Advanced Catalytic Enzyme System – ACES) has been developed at the Edgewood Chemical Biological Center (ECBC) for use by military services and civilian first responders. The ACES will contain enzymes for the detoxification of nerve agents (G and V) and related pesticides, sulfur mustard, bacterial cells, and anthrax spores. Catalytic enzymes are highly efficient, detoxifying many times their own weight of agent in seconds or minutes. The ACES is also non-corrosive, non-flammable, and environmentally safe. The ACES can fulfill a dual-use role as both decontaminant and fire extinguisher through the addition of environmentally safe fire-fighting materials.

INTRODUCTION

DECONTAMINATION REQUIREMENT

As stated in the Joint Science and Technology Chemical Biological Decontamination Master Plan:

> "Decontamination is defined as the process of removing or neutralizing a surface hazard resulting from a chemical/biological (CB) agent attack. Its purpose is to quickly restore battlefield operational tempo and logistics after a CB attack has occurred. CB agents require cumbersome protective measures that cause significant degradation of combat performance. Thus, decontamination capabilities are required to sustain operations in a CB contaminated environment, to ensure power projection capabilities, to clean up personnel and large areas for retrograde and resupply operations, and to reconstitute individual equipment, vehicles, and weapon platforms. The objective of decontamination technology advancement efforts is to develop systems that are rapid and effective in detoxifying CB agents, environmentally safe, do not impact the operation effectiveness of the equipment being decontaminated, and minimize the logistical impact on operations."

In addition to the traditional concept of dealing only with vehicles and equipment, it is also crucial to be able to decontaminate large areas such as logistics bases, airfields, ports, key command and control centers, and other fixed facilities. With the rising threat of terrorist attacks using CB agents or toxic industrial chemicals, decontamination of large civilian facilities is a major concern.

Current decontaminants are caustic and have the potential for causing materiel and environmental damage and personnel injury. This, and the fact that some are also flammable has made them inappropriate for shipboard use, on new, high performance aircraft, or on other non-hardened equipment.

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In addition, most are bulk liquids that require significant logistics/ storage capabilities. While some of these decontaminants are non-aqueous, they still require considerable amounts of water for pre-washing and post-application rinsing to prevent equipment corrosion. These realities further impinge on their applicability in dealing with interiors or large outdoor fixed sites that have been contaminated.

ENZYMATIC DECONTAMINATION

An appropriate mixture of enzymes and other natural products offers considerable advantages over other decontaminants. Being catalytic, the enzymes are highly efficient and can detoxify many times their own weight of agent in seconds or minutes. Since their first discovery during World War II [1], a variety of enzymes with activity against nerve agents and numerous organophosphorus pesticides has been identified. Properties of fluorine containing organophosphorus compounds and a review of the history of nerve agent-degrading enzymes can be found in a recent publication [2]. The three primary enzymes currently under investigation and intended for use in ACES are given in Table 1.

Table 1. Nerve Agent Detoxifying Enzymes

Enzyme	Original Source	Mol. Wt. (kDa)	Agent Activity
Organophosphorus Hydrolase (OPH)	Bacteria (*Pseudomonas diminuta*)	72	DFP > GF ≈ GB > GD > VX
Organophosphorus Acid Anhydrolase (OPAA)	Bacteria (*Alteromonas* sp. JD6.5)	58	GD > GF ≈ DFP > GB > GA
Diisopropylfluorophosphatase (DFPase)	Squid (*Loligo vulgaris*)	35	DFP > GF ≈ GB > GD > GA

OPH is an enzyme found in a number of bacterial isolates that has optimal activity against a variety of organophosphorus pesticides (originally called parathion hydrolase) in addition to its activity against nerve agents. While many researchers have studied OPH, the primary information on its structure and mode of activity has come from the laboratories of Frank Raushel and James Wild, both at Texas A&M University. The gene for this enzyme has been cloned, sequenced, and expressed in a number of prokaryotic and eucaryotic host organisms. The three-dimensional crystal structure of OPH also has been determined revealing that the native enzyme is a homodimer and contains two Zn^{2+} ions per subunit. The Co^{2+} substituted enzyme has greater activity on nerve agents and substrates with P-F and P-S bonds [3]. OPH is the only well characterized enzyme with catalytic activity against V-agents. Although several orders of magnitude slower than its paraoxon activity, the V-agent activity is significant and has been improved by an order of magnitude through site-directed mutagenesis carried out recently by several laboratories. While more research has been conducted on OPH compared to other chemical agent-degrading enzymes, its cellular function and native substrate remain unknown.

OPAA was originally identified in the obligate halophilic bacterium *Alteromonas* sp. JD6.5 that was isolated from Grantsville Warm Springs in Utah [4]. Unlike OPH, OPAA has very little activity against pesticides. The OPAA gene has been cloned, sequenced, and expressed at very high levels in *Escherichia coli* (up to 50% of cell protein). The enzyme can be freeze-dried and survive for many years at room temperature with no loss of activity. Crystallization studies are currently underway in the laboratory of Florante Quiocho, Baylor College of Medicine. From the amino acid sequence of OPAA and functional studies on a variety of dipeptides, it was identified as an X-Pro dipeptidase (or prolidase, EC 3.4.13.9) having nothing at all to do with phosphorus metabolism. When a model of the active site of OPAA (based on other peptidases) is examined, it can be seen that soman fits just as well as a natural substrate such as Leu-Pro. Through serendipity, it is ideally positioned for hydrolytic attack on the phosphorus atom. This class of enzymes can be found throughout nature in organisms as primitive and diverse as Archea and bacteria all the way up to humans. In all likelihood, they will be found in all organisms as part of their protein recycling system. Several other X-Pro dipeptidase from different organisms have been examined and all possess activity against G-type nerve agents, but much less than that of OPAA.

OPAA does not have activity against V-agents, but once the three-dimension structure is determined, site-directed mutagenesis will be undertaken to address this property. Kinetic studies have shown that VX inhibits the OPAA enzyme in a manner best modeled as competitive, strongly suggesting that VX does enter or bind in the active site even if it is not a substrate[5].

Of the nerve agent degrading enzymes, the squid DFPase has been studied for the longest period. Originally identified by Francis Hoskin in 1966, it was given the name DFPase in 1969 [6]. Most recently, extensive research has been conducted in the laboratory of Prof. Heinz Rüterjans, University of Frankfurt. The gene for the squid enzyme has been cloned, sequenced, and expressed in both *E. coli* and the yeast *Pichia pastoris* [7]. The squid-type DFPase has only been found in cephalopods, requires Ca^{2+} for activity and stability, and hydrolyzes DFP five times faster than soman. Its chemical and biological properties are completely different from those of all other types of DFPases as well as OPH and OPAA. To this day, it still appears to belong to a unique class of enzymes.

Unlike most chemical catalysts, enzymes with different properties and specificities can be mixed together in a single formulation. This takes advantage of their different activities and properties to provide as broad coverage as possible when in use. For example, if one enzyme is inhibited by a certain metal, other enzymes in the formulation with activity on the same substrate may be either stimulated or unaffected by it. This will ensure that no matter the type or quality of water used, enough of the various enzymes will be functioning to provide the necessary coverage. In addition, since most enzymes function best at pH values near neutrality, there are few, if any, compatibility or corrosion concerns as long as the material being decontaminated can tolerate water. Nerve agent-degrading enzymes are capable of functioning in a variety of water-based systems such as fire-fighting foams and sprays, aqueous degreasers, and commercial laundry detergents [8]. Examples of this are shown in Table 2.

Table 2. OPAA Activity on DFP in Possible Vehicles

Vehicle	Property	Conc. (%)	Rel. Act.
Control (Ammonium carbonate buffer, pH 8.7)	—	—	100
AFC-380 (Sandia Nat. Lab: Albuquerque, NM)	Blast containment foam	6	54
BioSolve® (Westford: Westford, MA)	Fire-fighting wetting agent	6	53
Fire Choke® (Fire Response: Houston, TX)	Class A fire-fighting foam	0.5	100
BV 406LF (FireFreeze: Rockaway, NJ)	Degreaser/cleaner	10	73
ColdFire® (FireFreeze: Rockaway, NJ)	Fire-suppressing agent	10	120
Odor Seal® (FireFreeze: Rockaway, NJ)	Odor removing wetting agent	10	102
Tide® Free (Proctor & Gamble)	Laundry detergent	0.05	108
CORNsolv (SOYsolv: Tiffin, OH)	Biodegradable solvent	1	121

Similar to commercial laundry detergents containing enzymes, an enzyme-based decontaminant will pose little or no health or environmental danger and leave no hazardous products requiring cleanup. Another major advantage is that an enzyme-based decontaminant would be provided as a dry powder that can be added to whatever water-based spray or foam systems are available to the user. This provides a significant reduction in the logistical burden (25-50 fold) as well as making use of existing equipment – both military and civilian. For example, to provide two million gallons of decontaminant for a major military engagement would require 11,000 tons of DS2. On the other hand, the equivalent amount of dry, enzyme-based decontaminant would weigh ~56 tons and have no special storage needs. This reduction in logistics burden is especially important for ships at sea. For example, when added to an aircraft carrier's Countermeasure Washdown System, less than 500 pounds of enzyme-based decontaminant would be required to treat the entire flight deck (1092 x 257 ft, ~200,000 ft^2, 4.5 acres) with 3" of foam.

While the enzyme-based system is aqueous, the overall result is actually reduction of the water usage and simplification of the decontamination operations. A primary goal is to use whatever water is available locally. In fact, the amount of water used with the enzyme-based system will be **less** than that

required with DS2. For example, under current decontamination protocols for an M1A1/M1A2 Abrams tank (120 m^2), the following materials are required:

320 gallons of water – Pre-wash
15 gallons of DS2 – Thorough Decon
80 gallons of water – Rinse

With an enzyme-based system, the pre-wash (to remove bulk agent) may also contain enzymes, thus beginning decontamination immediately as well as reducing/eliminating the agent that is being removed. Because of the non-corrosive nature of the enzyme-based system, the rinse step may be eliminated.

MUSTARD CHALLENGE

A major limiting factor in the development of ACES has been the problem of dealing with sulfur mustard (HD). Because it is essentially insoluble in water, making it available to aqueous hydrolytic enzymes is a significant challenge. If mustard can be solubilized, its spontaneous hydrolysis rate to form the relatively non-toxic thiodiglycol (TDG) is quite fast. Therefore, many approaches have been looked at to accomplish this goal. Several detergents have been tested to determine their effects on mustard hydrolysis and all were found to significantly inhibit hydrolysis [9]. The simplest explanation may be that the hydrophobic tails of the detergent micelles sequester mustard droplets from the surrounding aqueous environment. Alternatively, high concentrations of organic solvents can be used, but then one is back to using large volumes of bulk decontaminant in conjunction with non-hydrolytic chemistries. In chemistry-based decontaminants, the most common method of dealing with mustard is through oxidation to its sulfoxide. Mustard sulfoxide is no longer a vesicant although it does retain systemic toxicity. If the oxidation is continued, the sulfoxide is converted to the sulfone that again causes severe blistering.

Three approaches have been taken to deal with mustard. Because of the initial lack of a mustard enzyme, the first approach was to identify chemicals that will enhance the solubilization of mustard but at the same time are compatible with the enzymes in the formulation. A variety of detergents, foams, phosphonium compounds, and quaternary ammonium compounds were examined to determine their efficacy [9]. Most of the materials had either little effect on the dechlorination rate or inhibited hydrolysis. However, several quaternary ammonium compounds did result in significant enhancement in the hydrolysis rate. One such compound, dodecyldimethyl(3-sulfopropyl)ammonium hydroxide (DDSAH) acts as a phase transfer catalyst. It not only increased the rate of mustard hydrolysis but also was shown to be compatible with OPH, OPAA and DFPase (Cheng and Rastogi, personal communication).

The second approach was to use mild chemical oxidants (such as those used in laundry detergents with enzymes) to convert mustard to its sulfoxide, which is much more water-soluble. A dehalogenase enzyme would then be used to convert the mustard sulfoxide to thiodiglycol sulfoxide, which is known to be non-toxic. Such a dehalogenase was identified in the bacteria *Sphingomonas paucimobilis* and is undergoing purification and characterization (Elashvili, personal communication).

The third approach, the search for enzymes that could directly attack mustard has continued, especially among those known to be able to dechlorinate chlorinated alkanes. A bacterial enzyme with this ability was recently identified and shown to significantly enhance the hydrolysis rate of mustard even in the absence of any solvent [10]. One explanation for the enzyme's ability to deal with insoluble substrates may be due to the presence of hydrophobic amino acids around its active site. This could also explain its interaction with mustard.

BW AGENTS

A variety of approaches will be used in dealing with the decontamination of BW agents. Several of the non-enzymatic components of the proposed system (DDSAH and ColdFire®) have been shown to give

significant killing of non-pathogenic *B. anthracis* cells and other simulants (Harvey and Rastogi, personal communication). Enzymes that may be incorporated into the formulation will include commercial ones such as lysozyme and others that are currently under development. Some of these are oxidative fungal enzymes currently under development by Novozymes A/S (Bagsvaerd, Denmark) with activity against both cells and spores, and a bacteriophage enzyme (PlyG Lysin) that is specific for *B. anthracis* cells [11].

Other natural products such as plant essential oils, bacteriocins, and biosurfactants are under investigation at ECBC and show considerable promise. Many of these materials are already foods or approved food additives. Several even have shown significant killing properties as vapors, making them of interest in the decontamination of sensitive equipment and building or vehicle interiors. It is felt that by incorporating a variety of these materials, the likely synergistic effects will result in a highly potent BW agent decontaminant that will be innocuous to higher forms of life.

FIRE-FIGHTING CAPABILITIES

The ACES has the potential for a dual role as both decontaminant and fire extinguisher. The fire fighting properties of ACES are due to the inclusion of ColdFire® and Fire Choke®. ColdFire® is a fire extinguishing agent derived from a variety of vegetable extracts that combines rapid fire knockdown, a remarkable ability to remove heat, and environmental safety. Studies conducted by FireFreeze in conjunction with ECBC showed that the addition of enzymes and ammonium carbonate buffer to ColdFire® had no negative effects on its ability to extinguish fires. In addition, most of the enzymes that will be incorporated into ACES are significantly stimulated and/or stabilized by ColdFire®.

Fire Choke® is a Class A fire fighting foam that is also a very effective knockdown agent, compatible with the enzymes, environmentally safe, and certified for use on military aircraft. The combination of ColdFire® and Fire Choke® gives an extremely potent fire suppressing/extinguishing foam system. Both materials are now available in dry formulations, thus simplifying the production of a single, dry powder system and further improving the logistics benefits. A concentrated liquid formulation is also possible, but it will require significant developmental efforts to stabilize the various enzymes in such a system. It should be emphasized that for commercial use, the type of foam used (if any) will the decision of the user. The goal is for the enzymes to function in all major foam systems currently on the market.

INITIAL FORMULATION

The initial formulation of ACES will likely consist of the following components:

Nerve Agents and Pesticides: Organophosphorus acid anhydrolase (OPAA) [12], Organophosphorus hydrolase (OPH) [13], Recombinant DFPase from squid (from Roche) [7]
Sulfur Mustard: Bacterial HD hydrolase [10], DDSAH [9]
BW Agents: Oxidative fungal enzymes (from Novozymes), Lysozyme (commercial)
Buffer: Ammonium carbonate
Fire-Fighting Components: ColdFire®, Fire Choke® or an equivalent Class A foam

The packaging of ACES would be in sizes that will conform to the types of equipment in which it will be used. This could range from one-ounce packets that individual soldiers could carry, up to 50-pound pails for use with large pumping systems.

LEVERAGING

Collaborative work is currently underway with Genencor International in regard to large-scale production of the OPAA/OPH enzymes. Under a cooperative research and development agreement (CRADA) being negotiated with Genencor, other enzymes will be included in this scale-up program.

Development of enzyme-based decontamination systems is also the goal of NATO Project Group 31 (PG/31), "Non-Corrosive, Biotechnology-Based Decontaminants for CBW Agents." NATO PG/31 consists of France (FR), Germany (GE), Italy (IT), Turkey (TU), the United Kingdom (UK) and the United States (US) as lead nation. The Czech Republic (CZ), Denmark (DA), and Hungary (HU) are currently pursuing membership. Among the member nations, decontamination research is underway dealing with not only nerve agent and mustard but also organophosphorus and carbamate pesticides, BW agents, and potential decontaminant application systems.

Standardized panel testing of single enzyme systems under the auspices of NATO PG/31 has consistently given excellent results as shown in Table 3.

Table 3. NATO PG/31 Enzyme Decon Tests

Year	Enzyme	Agent	Surface	Decon Efficacy of Plate (%)			
				15 min	20 min	30 min	40 min
1997 (FR)	OPAA (CF)	GD	PU	99.9		>99.9	
	DFPase (Silv-EX)	GD	PU	>99.9		>99.9	
	OPAA (Silv-EX)	GD	PU	>99.9		>99.9	
1998 (FR)	OPAA (CF + Silv-EX)	GD	PU	99.3		99.7	
	DFPase (buffer)	GD	PU	99.6		99.6	
1999 (FR)	OPH (CF)	**VX**	PU		>99.9		>99.9
	OPAA (Tide)	GD	PU	99.6		99.7	
1999 (GE)	OPAA (UK μEmul)	GD	**Alkyd (3 hr)**	>99.9		>99.9	
2000 (UK)	OPAA (UK μEmul)	**TGD**	PU (1 hr)			99.7	
	OPAA (CF)	**TGD**	PU (1 hr)			99.7	
2002 (FR)	DFPase in Eco-Foam	GD	PU	>99.9		>99.9	

CF = ColdFire; Eco-Foam = foam; μEmul = microemulsion; PU = polyurethane; Silv-EX = foam

MEDICAL APPLICATIONS

Potentially, an enzyme-based decontaminant will be benign enough to be used directly on the skin of personnel or casualties. It could be employed in shower systems and other wash down operations as well in sponge-based applicators for personal decontamination. The ability to permanently immobilize OPH and other enzymes in polyurethane foams and maintain their catalytic activity has been demonstrated [14].

In addition to external use, there also is increasing interest in the use of catalytic enzymes for prophylaxis and/or treatment for nerve agent exposure. Current treatment for nerve agent exposure is injection of atropine and/or pralidoxime (2-PAM). While effective, atropine and 2-PAM treatment causes side effects that can incapacitate the victim for days. Liposome-encapsulated OPAA has been shown to provide significant survival enhancement in mice [15]. OPAA in stabilized liposomes (SL) was given intravenously through tail vein one hour prior to receiving DFP. The results are summarized in Table 4.

Not only does the enzyme provide similar protection as atropine or 2-PAM alone, a significant enhancement is seen when all three are combined. With the atropine and 2-PAM protecting the active site of acetylcholinesterase and the circulating enzyme detoxifying the DFP in the bloodstream, a 3.3-fold synergistic effect is obtained increasing the LD_{50} from 4.2 to 98.6 mg/kg. This offers the possibility that with the addition of even more active enzymes, the amount of atropine and 2-PAM used could be reduced, thus still providing a significant degree of protection while reducing the debilitating side effects.

Table 4. Protective Effect of OPAA in Mice

Atropine (10 mg/kg ip)	2-PAM (90 mg/kg ip)	OPAA-SL (20-30 units iv)	LD_{50} (mg/kg)	Potency Ratio
—	—	—	4.2	—
+	—	—	5.7	1.34
—	+	—	7.7	1.83
+	+	—	29.3	**6.98**
—	—	+	9.6	2.29
+	—	+	18.9	4.50
—	+	+	21.1	5.02
+	+	+	98.6	**23.48**

While these results are quite impressive, even more significant enhancement has been seen with liposome encapsulated OPH against paraoxon[16], the highly toxic metabolic product of the pesticide parathion. In this case, the protective effect was increased to over **1000 times the LD_{50}**. This offers considerable promise in dealing with the huge number of organophosphorus pesticide poisonings and deaths that occur worldwide every year. In addition to these uses, enzymes could play several other roles in the agricultural environment. These could include the cleanup of containers and equipment used in pesticide application as well as the post-harvest washing of fruits and vegetables to remove any trace pesticide contamination.

CONCLUSION

The ACES is intended to eventually replace Decontaminating Solution 2 (DS2), supertropical bleach (STB) and other current decontaminants as either an improvement to the Joint Service Family of Decon Systems (JSFDS) or as part of the Joint Service Superior Decon Solution (JSSDS) Program that was scheduled for production in FY07-08. However, introduction of a commercial version of ACES is intended to occur in 2003. Discussions with potential marketing/production firms are now underway.

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